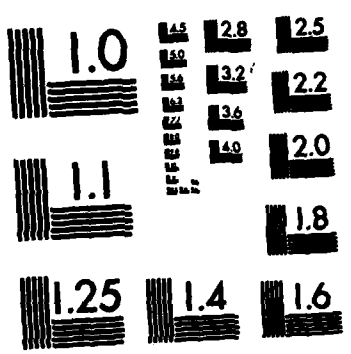
AD-A125 149 THE MECHANISM OF BINDING AND SECRETION OF ALKALINE PHOSPHATASE ACROSS THE.. (U) DEFENCE RESEARCH ESTABLISHMENT SUFFIELD RALSTON (ALBERTA) 1/1

UNCLASSIFIED A R BHATTI ET AL. NOV 82 DRES-SM-1068 F/G 6/1 NL



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

UNCLASSIFIED

UNLIMITED DISTRIBUTION

3

DRES

SUFFIELD MEMORANDUM

NO. 1068

THE MECHANISM OF BINDING AND SECRETION OF ALKALINE PHOSPHATASE ACROSS THE CELL MEMBRANES OF *PSEUDOMONAS AERUGINOSA* (U)

by

A.R. Bhatti and J.M. Ingram*

ACN No. 16A10

November 1982

* Department of Microbiology, MacDonald Campus of McGill University
Ste Anne de Bellevue, P.Q., Canada

CANADA

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD
RALSTON ALBERTA

SUFFIELD MEMORANDUM NO. 1068

THE MECHANISM OF BINDING AND SECRETION OF AKALINE PHOSPHATASE ACROSS THE CELL MEMBRANES OF *PSEUDOMONAS AERUGINOSA* (U)

by

A.R. Bhatti and J.M. Ingram*

ACN No. 16A10

* Department of Microbiology, MacDonald Campus of McGill University
Ste Anne de Bellevue, P.Q., Canada

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD
RALSTON ALBERTA

SUFFIELD MEMORANDUM NO. 1068

THE MECHANISM OF BINDING AND SECRETION OF ALKALINE PHOSPHATASE ACROSS THE CELL MEMBRANES OF *PSEUDOMONAS AERUGINOSA* (U)

by

A.R. Bhattti and J.M. Ingram

ABSTRACT

During growth, pH of the medium decreases resulting in the inactivation of cell-free and surface-bound alkaline phosphatase, whereas periplasm-located alkaline phosphatase is not affected. The decreased pH of the medium induced permeability changes in the outer cell wall which resulted in the complete release of alkaline phosphatase after sucrose extraction. The permeability change of the outer cell wall is pH dependent and reversible. The periplasm-located alkaline phosphatase remains constant during growth of *Pseudomonas aeruginosa*. A hypothetical model of the mechanism for the release of periplasm-located proteins into the growth medium during growth is presented.

(U)

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD
RALSTON ALBERTA

SUFFIELD MEMORANDUM NO. 1068

THE MECHANISM OF BINDING AND SECRETION OF ALKALINE PHOSPHATASE ACROSS THE CELL MEMBRANES OF *PSEUDOMONAS AERUGINOSA* (U)

by

A.R. Bhattti and J.M. Ingram

INTRODUCTION

Alkaline phosphatase (orthophosphoric monoester phosphorylase, EC 3.1.3.1) is of ubiquitous occurrence in animal, plant and insect tissues as well as in microorganisms. A survey of the literature reveals very little information on bacterial alkaline phosphatase until 1959, but since the discovery of alkaline phosphatase in *Escherichia coli* (11, 18, 10), this topic has attracted a great deal of interest. It has been studied in several other bacterial species (1). Many bacteria are known to secrete extracellular enzymes into the external medium. In gram-positve strains, these proteins appear in the culture medium, whereas in gram-negative strains the polypeptides are retained in a region located between the inner and outer membrane, which is called the periplasmic space. An obvious problem that has long been recognized for these proteins secreted outside of the cytoplasm is: what is the mechanism that allows these proteins to cross the highly hydrophobic layer constituted by the inner membrane and what is the basis for the selective secretion of these proteins? Bacterial systems are convenient models for examining these problems. *Pseudomonas aeruginosa* synthesizes an inducible periplasm-located alkaline phosphatase in inorganic phosphate (Pi) limited medium (6). The

enzyme is released to the medium during growth (2), and has been shown by electron microscopy to be localized both in the periplasm and on the outer surface of the cell (12). The enzyme was purified to homogeneity and shown to be a dimer (8). The native dimer is resistant to heat and proteolysis (8) and is dissociated by acid pH (pH 5.0) whereas the monomer is unstable to heat and is sensitive to trypsin digestion (12). The enzyme is released completely from whole cells after suspension in 0.2 M $MgCl_2$ and a fractional amount is released by suspension in 20% sucrose (6).

The present study was undertaken to demonstrate that, during growth, the pH of the medium decreases, resulting in the inactivation of cell-free and surface-bound alkaline phosphatase whereas periplasm-located alkaline phosphatase is not affected. The decreased pH of the medium induced permeability changes in the outer cell wall which results in the complete release of alkaline phosphatase after sucrose suspension. The permeability change of the outer cell wall is pH dependent and reversible. Finally, the quantity of periplasm-located alkaline phosphatase remains constant during growth of *P. aeruginosa*. A hypothetical model on the mechanism for the secretion of periplasm-located proteins into the growth medium during growth is presented.

MATERIALS AND METHODS

Organism and Culture Conditions

P. aeruginosa (ATCC 9027) was grown in inorganic phosphate deficient medium of the following composition as previously described (3): 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 0.5% proteose peptone (Difco), and 0.0016 M $MgCl_2 \cdot 7H_2O$. The medium, without glucose and $MgCl_2$, was prepared, and the pH was adjusted to 6.8 with concentrated HCl. The medium was autoclaved and filtered, and the pH was then readjusted to 6.8, if necessary, and reautoclaved. Before inoculation, 2.0 mL of 25% glucose and 1.0 mL of 0.16 M $MgCl_2 \cdot 7H_2O$ solution per 100 mL of medium were added aseptically. This Pi limited medium is necessary to depress the synthesis of alkaline phosphatase (6). Growth studies were subsequently conducted at the temperatures specified in the particular experiment. An inoculum of 1 mL from a 7- to 10-h culture was used per 100 mL of medium, and growth was carried out by incubation in a gyratory shaker (psychrotherm shaker, New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C or other specified temperatures.

Preparation of Samples for Enzyme Activity Estimation

Equal volumes of *P. aeruginosa* cultures were centrifuged (10,000 × g) and the pellets were suspended in equal volumes of 20% sucrose in 10 mM Tris-HCl buffer, pH 8.4, and 200 mM $MgCl_2$ in the same buffer. After 10 min of incubation at room temperature, the cells were centrifuged. The supernatant fluids obtained from sucrose-extracted cells and $MgCl_2$-extracted cells were assayed for surface-bound alkaline phosphatase (SBE) and the total alkaline phosphatase activity (TE) associated with the cells, i.e., SBE as well as periplasm alkaline phosphatase (6). PE was obtained by subtracting SBE from TE.

Enzyme Assay

Enzyme assays for alkaline phosphatase and glucose-6-phosphate dehydrogenase were performed as described previously (6, 2).

RESULTS AND DISCUSSION

The Effect of pH Changes During Growth on the Release of Alkaline Phosphatase Activity

The effect of pH changes during growth on the release of periplasm-located alkaline phosphatase is illustrated in Fig. 1A. During the initial stage of alkaline phosphatase synthesis (4 – 5 h), no detectable SBE (curve 2) was extracted with sucrose. Cell-free enzyme (CFE, Curve 1) activity increased to 44 units after 6 h, and this was followed by a decrease in CFE activity to 3 units after 11 h of growth. The decrease in enzyme activity was observed previously (12) and was due to the decreased pH (6.86 to 5.2) of the culture medium.

When growth was allowed to proceed for 24 h, there was an increase in the pH of the culture medium (pH 5.2 to 5.8, Fig. 1B) which results in an increase in the CFE activity (3 units to 9 units, Fig. 1A). The increase in CFE alkaline phosphatase activity is probably due to dimerization of pre-existing monomers in the culture fluid or incomplete monomerization, at pH 5.8, of newly-released alkaline phosphatase. The quantity of PE increased from 6 units to 73 units (curve 3) after 7 h of growth. As growth proceeded (8 to 11 h), the level of PE decreased from 50 units to −6 units, and

the pH of the growth medium decreased from pH 6.0 to 5.2. Further growth from 11 h to 24 h resulted in an increase of 15 units in PE activity (curve 3, Fig. 1A). The SBE activity increased from 0 to 72 units within 5 to 10 h of growth and then decreased from 70 to 11 units during the 11th and 24th h of growth (Curve 2, Fig. 1A). Both sucrose and $MgCl_2$ supernatant fluids obtained from cells during growth between 6 and 24 h gave no detectable glucose-6-phosphate dehydrogenase activity indicating that cytoplasmic proteins were not released by these procedures. Phase contrast microscopy revealed that the cells were intact but plasmolyzed.

The results of the present study support the findings of Cheng *et al.* (7), which suggested that alkaline phosphatase is bound by electrostatic forces mediated perhaps by Mg^{2+} since it was found that during the later stages of growth, when the pH of the culture medium dropped below 6.0, 10% sucrose extracted TE. Therefore, low pH (high proton concentration) must decrease the electrostatic forces which bind alkaline phosphatase to the outer cell wall structure thereby aiding the sucrose extraction of TE. Conversely, at later stages of growth, when the pH of the culture medium increased, sucrose was ineffective in releasing TE alkaline phosphatase. Therefore, it is concluded that, unlike low pH as mentioned above, high pH re-establishes or increases the electrostatic forces in the outer cell wall thus preventing the extraction of TE by suspension in sucrose (4). Under these conditions, cells appear plasmolyzed but essentially intact after the sucrose extraction procedure, and consequently, plasmolysis effects alone are unable to account for release of TE by sucrose.

The effect of a temperature shift during growth on SBE and PE is illustrated in Fig. 2A. Surface bound enzyme (curve 1) and PE (curve 2) activities increased before the temperature shift (indicated by an arrow) from 37 to 46°C. At 46°C, the PE activity increased in the first hour followed, subsequently, by a slight increase in absorbance at 600 nm (growth) (Fig. 2B). The SBE activity increased gradually to 63 units after 10 h of growth and decreased to 48 units after 11 h. This decreased SBE activity occurs when the pH of the culture medium declines. There is also a signficant increase in the SBE observed after 9 h of growth (curve 3). It appears probable that, after the shift to 46°C, some of the CFE may rebind to the cell surface which relates to the decrease in CFE and a subsequent increase in SBE, while the PE remains almost constant. Alternately, this relative increase in SBE activity may be due to the fact that CFE is inactivated, whereas additional PE is synthesized and transported to the outer surface, while PE activity remains constant. It should be stressed again that no SBE appears during early stages of

enzyme synthesis indicating that the sequence of alkaline phosphatase localization is firstly, in the peri-plasm, then the outer cell wall surface and, finally, culture filtrate, i.e., apparent secretion.

It has been observed that the pH of cultures growing at 46°C does not change significantly when compared to cultures growing at 37°C (3) and, therefore, temperature shift experiments were pursued further to substantiate the effect of pH on the permeability of the outer cell wall in connection with the release of TE. After temperature shift from 37 to 46°C, the pH of the culture did not change significantly (Fig. 2B) and the amount of PE remained almost constant. Sucrose did not induce a release of TE from the cell at any stage of growth after the shift as shown in the previous experiments (6). The marked increase in PE activity in the first hour after the temperature shift is presumably due to the dimerization of monomers which were already synthesized by actively growing cells at 37°C (3). The data also show that PE activity decreases after the temperature shift whereas CFE continues to increase. This result suggests that PE eventually gives rise to CFE. Since sucrose did not increase the extraction of PE activity, the outer cell wall permeability function is still intact. Therefore, the appearance of PE activity in CFE must be the result of a normal physiological "secretion" process.

Molecular Mechanism for the Release of Alkaline Phosphatase

In order to study the molecular mechanisms which are involved in the secretion of proteins across biological membranes, it is useful to have a simple model system. Based on the results of the present study and the work of others, a hypothetical model on the release of alkaline phosphatase and other perplasm located proteins is presented (Fig. 3).

The results reported in the present study support the theory proposed by Kung and Henning (1972), that a limiting and constant number of binding sites in the cell envelope exist for each type of protein.

The precursor of monomer alkaline phosphatase is synthesized on the membrane bound polyribosomes (5, 19). This completed nascent monomer, after traversing the cytoplasmic membrane, becomes accessible to a protease located at the outer side of the cytoplasmic membrane (13) which then releases the enzyme monomer into the periplasm. Since alkaline phosphatase monomers are very susceptible to proteolytic cleavage (16), the monomers fold up and dimerize before they can be degraded. Pages *et al.* (15), in

their study, have reported the evidence for this limited proteolytic degradation of alkaline phosphatase monomers.

The release of periplasm-located enzymes to the growth medium during growth of *P. aeruginosa*, therefore, may be due to the fact that the binding sites become saturated and newly synthesized enzyme (PE) and lipopolysaccharide (LPS) molecules displace the "older" molecules to the outer surface of the cell wall (SBE), and eventually into the culture filtrate (CFE). This model would explain the finding that substantial amounts of other periplasm-located enzymes are released into the medium during growth, whereas, these enzymes are not released significantly by any of the biochemical procedures used (2).

REFERENCES

1. Bhatti, A.R. (1974). Purification and properties of the alkaline phosphatase of *Serratia marcescens*. Arch. Microbiol. **95**: 255 – 266.

2. Bhatti, A.R., I.W. DeVoe and J.M. Ingram (1976). The release and characterization of some periplasm-located enzymes of *Pseudomonas aeruginosa*. Can. J. Microbiol. **22**: 1425 – 1429.

3. Bhatti, A.R., I.W. DeVoe and J.M. Ingram (1976). Cell division in *Pseudomonas aeruginosa*: Participation of alkaline phosphatase. J. Bacteriol. **126**: 400 – 409.

4. Bhatti, A.R. and J.M. Ingram (1982). The binding and secretion of alkaline phosphatase by *Pseudomonas aeruginosa*. FEMS Microbiol. Letters. **13**: 353 – 356.

5. Cancedda, R. and M.J. Schlesinger (1974). Localization of polyribosomes containing alkaline phosphatase nascent polypeptides on membranes of *Escherichia coli*. J. Bacteriol. **117**: 290 – 301.

6. Cheng, K.-J., J.M. Ingram and J.W. Costerton (1970). Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and pH. J. Bacteriol. **104**: 748 – 753.

7. Cheng, K.-J., J.M. Ingram and J.W. Costerton (1970). Alkaline phosphatase localization and spheroplast formation of *Pseudomonas aeruginosa*. Can. J. Microbiol. **16**: 1319 – 1324.

8. Day, D.F. and J.M. Ingram (1973). Purification and characterization of *Pseudomonas aeruginosa* alkaline phosphatase. Can. J. Microbiol. **19**: 1225 – 1233.

9. Day, D.F. and J.M. Ingram (1975). *In vitro* studies of an alkaline phosphatase cell wall complex from *Pseudomonas aeruginosa*. Can. J. Microbiol. **21**: 9 – 16.

10. Garen, A. and C. Levinthal (1960). A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *Escherichia coli*. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta. **38**: 470 – 483.

REFERENCES (Cont'd)

11. Horiuchi, T., S. Horiuchi and D. Mizuno (1959). A possible negative feedback phenomenon controlling formation of alkaline phosphomonoesterase in *Escherichia coli*. Nature **183**: 1529 – 1530.

12. Ingram, J.M., K.-J. Cheng and J.W. Costerton (1972). Alkaline phosphatase of *Pseudomonas aeruginosa*. II. The mechanism of secretion and release of the enzyme from whole cells. Can. J. Microbiol. **19**: 1407 – 1415.

13. Inouye, H. and J. Beckwith (1977). Synthesis and processing of an *Escherchia coli* alkaline phosphatase precursor *in vitro*. Proc. Natl. Acad. Sci. **74**: 1440 – 1444.

14. Kung, H. and V. Henning (1972). Limiting availability of binding sites for dehydrogenases on the cell membrane of *Escherichia coli*. Proc. Nat. Acad. Sci. **69**: 925 – 929.

15. Pages, J.M., M. Piovant, S. Varenne and C. Lazdunski (1978). Mechanistic aspects of the trnasfer of alkaline phosphatase across the cytoplasmic membrane in *Escherichia coli*. Eur. J. Biochem. **86**: 589 – 602.

16. Schlesinger, M.J. (1965). The reversible dissociation of the alkaline phosphatase of *Escherichia coli*. J. Biol. Chem. **240**: 4293 – 4298.

17. Schlesinger, M.J. and K. Barrett (1965). The reversible dissociation of the alkaline phosphatase of *Escherichia coli*. I. Formation and reactivation of subunits. J. Biol. Chem. **240**: 4284 – 4292.

18. Torriani, A. (1960). Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. Biochem. Biophys. Acta. **38**: 460 – 469.

19. Varenne, S., M. Piovant, J.M. Pages and C. Lazdunski (1978). Evidence for synthesis of alkaline phosphatase on membrane-bound polysomes in *Escherichia coli*. Eur. J. Biochem. **86**: 603 – 606.

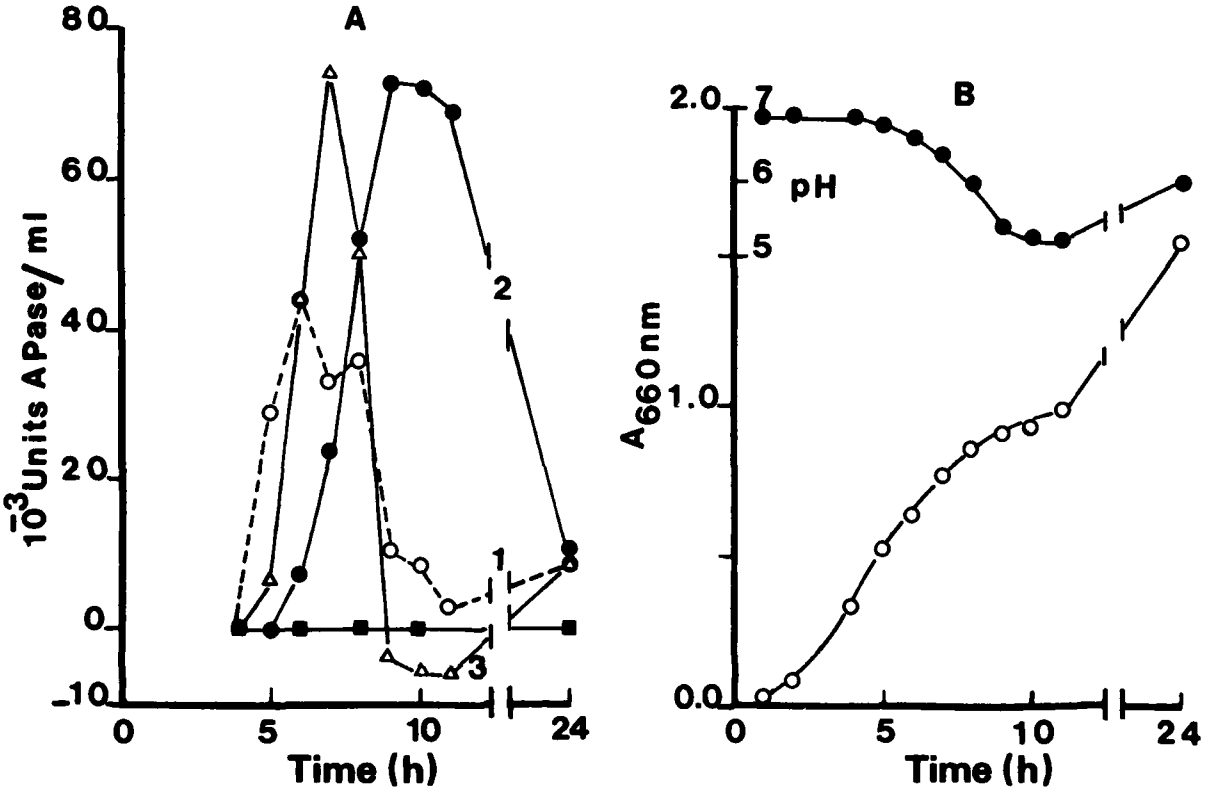


Figure 1. The effect of pH changes during growth on the release of alkaline phosphatase and glucose-6-phosphate dehydrogenase.

P. aeruginosa was cultivated at 37°C and at the indicated time intervals samples were withdrawn and analyzed for growth, pH changes, alkaline phosphatase and glucose-6-phosphate dehydrogenase activities as described in the text. Symbols: A; CFE (O), SBE (●), PE (Δ) and glucose-6-phosphate dehydrogenase (■). B; Growth A_{660} nm (O), pH (●).

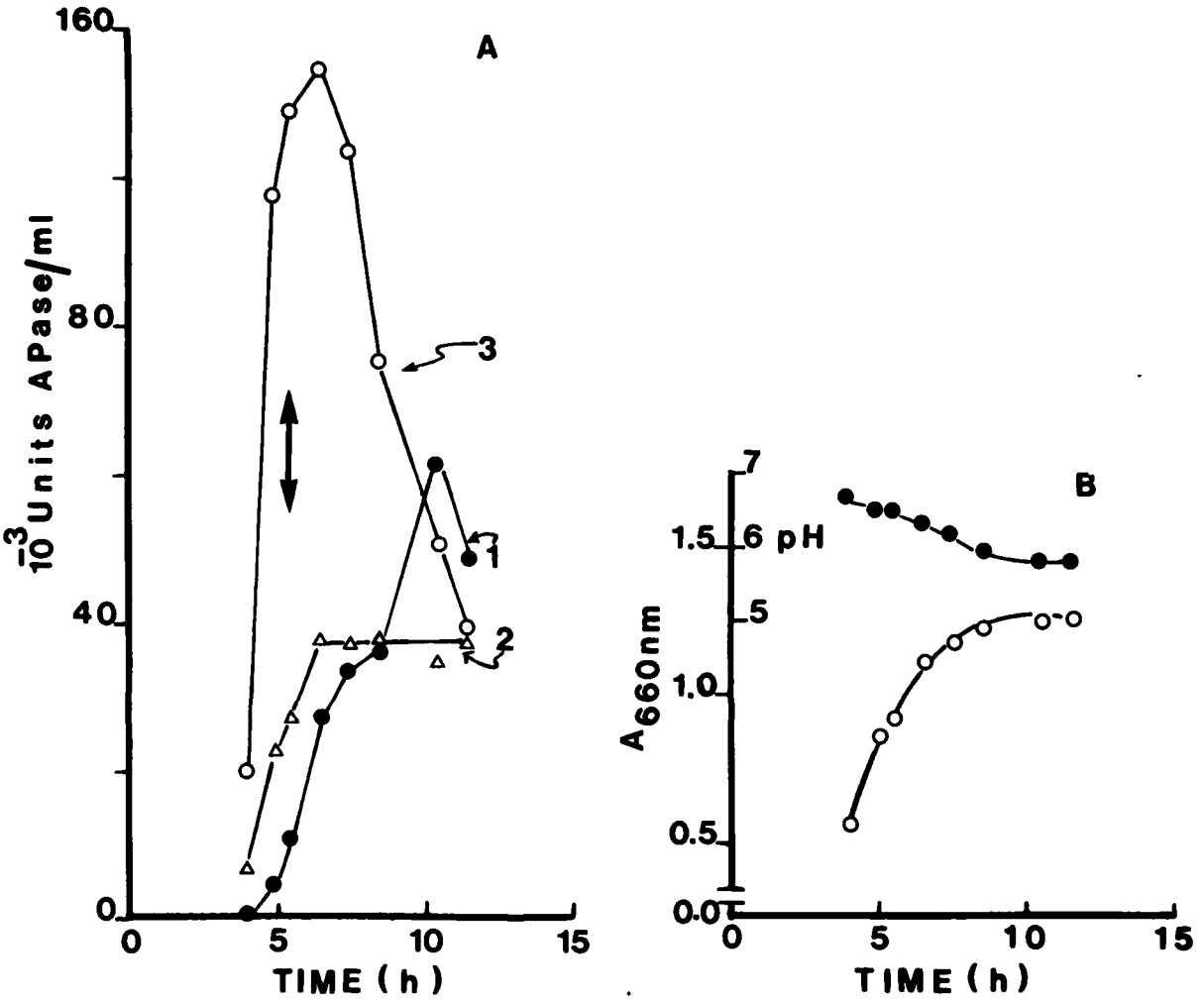


Figure 2. The effect of a temperature shift from 37°C to 46°C on the release of alkaline phosphatase activity.

A culture of *P. aeruginosa* was cultivated initially at 37°C and shifted to 46°C (as indicated by an arrow). Samples were withdrawn at regular intervals and analyzed for growth, pH, APase activities as described in the text. Symbols: A and B same as Fig. 1 A, B.

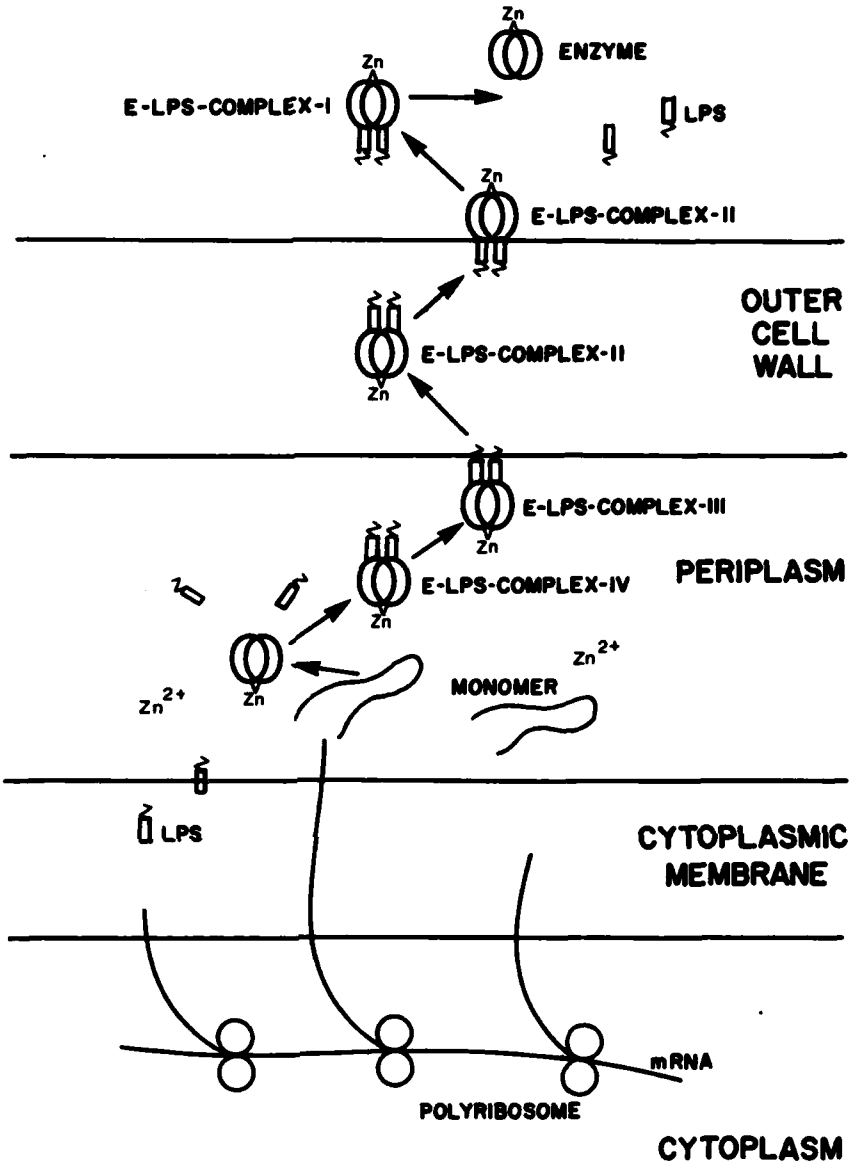


Figure 3

Schematic illustration of specific binding sites and release of periplasm-located APase. Polypeptide is synthesized on the polyribosomes and a protease present at the outer side of the cytoplasmic membrane [19] released the polypeptide to the periplasm. In the periplasm two nascent subunits or monomers polymerize in the presence of Zn^{+2} [17] to give active alkaline phosphatase (PE) (or other periplasm-located enzymes). This active alkaline phosphatase dimer complexes with lipopolysaccharide (LPS) [9] such that the enzyme-LPS complex I becomes part of the inner surface of the outer cell wall. The process is repeated and due to a limited number of sites, the original enzyme LPS-complex I migrates to the outer aspects of the cell wall and the new enzyme-LPS complex II takes its place. On further repitition of the process an anzyme-LPS complex III replaces the enzyme-LPS complex II (SBE) in the outer surface of the cell wall and the enzyme-LPS complex I is released to the growth medium as CFE.

Unclassified
This Sheet Security Classification

DOCUMENT CONTROL DATA - R & D
(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1 ORIGINATING ACTIVITY DRES	2a. DOCUMENT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3 DOCUMENT TITLE
The Mechanism of Binding and Secretion of Alkaline Phosphatase across the Cell Membranes of PSEUDOMONAS AERUGINOSA.

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, middle initial)
A. R. Bhatti and J.M. Ingram

6. DOCUMENT DATE November 1982	7a. TOTAL NO. OF PAGES 15	7b. NO. OF REFS 19
8a. PROJECT OR GRANT NO. 16A10	9a. ORIGINATOR'S DOCUMENT NUMBER(S) SM 1068	
8b CONTRACT NO.	9b. OTHER DOCUMENT NO.(S) (Any other numbers that may be assigned this document)	

10. DISTRIBUTION STATEMENT
Unlimited

11. SUPPLEMENTARY NOTES	12. SPONSORING ACTIVITY

13. ABSTRACT

During growth, pH of the medium decreases resulting in the inactivation of cell-free and surface-bound alkaline phosphatase, whereas periplasm-located alkaline phosphatase is not affected. The decreased pH of the medium induced permeability changes in the outer cell wall which resulted in the complete release of alkaline phosphatase after sucrose extraction. The permeability change of the outer cell wall is pH dependent and reversible. The periplasm-located alkaline phosphatase remains constant during growth of Pseudomonas aeruginosa. A hypothetical model of the mechanism for the release of periplasm-located proteins into the growth medium during growth is presented.

DSIS
79-070

KEY WORDS

Alkaline Phosphatase

Periplasm

P. aeruginosa

INSTRUCTIONS

1. ORIGINATING ACTIVITY Enter the name and address of the organization issuing the document.

2a. DOCUMENT SECURITY CLASSIFICATION Enter the overall security classification of the document including special warning terms whenever applicable

2b. GROUP Enter security reclassification group number. The three groups are defined in Appendix 'M' of the DRB Security Regulations.

3. DOCUMENT TITLE Enter the complete document title in all capital letters. Titles in all cases should be unclassified. If a sufficiently descriptive title cannot be selected without classification, show title classification with the usual one capital-letter abbreviation in parentheses immediately following the title.

4. DESCRIPTIVE NOTES Enter the category of document, e.g. technical report, technical note or technical letter. If appropriate, enter the type of document, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered

5. AUTHOR(S) Enter the name(s) of author(s) as shown on or in the document. Enter last name, first name, middle initial. If military, show rank. The name of the principal author is an absolute minimum requirement

6. DOCUMENT DATE Enter the date (month, year) of Establishment approval for publication of the document.

7a. TOTAL NUMBER OF PAGES The total page count should follow normal pagination procedures, i.e., enter the number of pages containing information

7b. NUMBER OF REFERENCES Enter the total number of references cited in the document.

8a. PROJECT OR GRANT NUMBER If appropriate, enter the applicable research and development project or grant number under which the document was written.

8b. CONTRACT NUMBER If appropriate, enter the applicable number under which the document was written

9a. ORIGINATOR'S DOCUMENT NUMBER(S) Enter the official document number by which the document will be identified and controlled by the originating activity. This number must be unique to this document

9b. OTHER DOCUMENT NUMBER(S). If the document has been assigned any other document numbers (either by the originator or by the sponsor), also enter this number(s).

10. DISTRIBUTION STATEMENT: Enter any limitations on further dissemination of the document, other than those imposed by security classification, using standard statements such as:

(1) "Qualified requesters may obtain copies of this document from their defence documentation center."

(2) "Announcement and dissemination of this document is not authorized without prior approval from originating activity."

11. SUPPLEMENTARY NOTES Use for additional explanatory notes.

12. SPONSORING ACTIVITY. Enter the name of the departmental project office or laboratory sponsoring the research and development. Include address.

13. ABSTRACT Enter an abstract giving a brief and factual summary of the document, even though it may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall end with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (TS), (S), (C), (R), or (U)

The length of the abstract should be limited to 20 single-spaced standard typewritten lines, 7½ inches long.

14. KEY WORDS Key words are technically meaningful terms or short phrases that characterize a document and could be helpful in cataloging the document. Key words should be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location, may be used as key words but will be followed by an indication of technical context.